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CONTRACT NO: DAMD17-87-C-7177

TITLE: DEVELOPMENT OF A NEW CHEMOTHERAPY FOR HUMAN AFRICAN TRYPANOSOMIAS USING AN ANI AL MODEL: SURAMIN WITH DL-ALPHA-DIFLUOROMETHYLORNITHINE (DFMO)

SUBTITLE: Chemotherapy for African Trypanosomiasis by Polyamine Synthesis Inhibition

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REPORT DATE: September 14, 1988

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION: Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION / AVAILABILITY OF REPORT: Approved for public release; distribution unlimited
2b. DECLASSIFICATION / DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION: New York University Medical Center; 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code): Department of Medical and Molecular Parasitology, New York, New York 10010	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING / SPONSORING ORGANIZATION: U.S. Army Medical Research & Development Command; 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: Contract No. DAMD17-87-C-7177
8c. ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, Maryland 21702-5012	10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO.
61102A	61102BS13	AF	061

11. TITLE (Include Security Classification)
Development of a New Chemotherapy for Human African Trypanosomias Using an Animal Model: Suramin with DL-Alpha-Difluoromethylornithine (DFMO)

12. PERSONAL AUTHOR(S)
Allen B. Clarkson, Jr., Ph.D.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual Report	FROM 8/15/87 TO 8/14/88	1988 September 14	22

16. SUPPLEMENTARY NOTATION
Subtitle: Chemotherapy for African Trypanosomiasis by Polyamine Synthesis Inhibition

17. COSATI CODES		
FIELD	GROUP	SUB-GROUP
06	03	
06	15	

18. SUBJECT TERMS (~~Continue on reverse if necessary and identify by block number~~)
African Trypanosomiasis; Trypanosoma Brucei Rhodesiense; DFMO; Difluoromethylornithine; Chemotherapy; Late Stage Disease ~~RA I~~ TS

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Towards developing a new chemotherapy for African trypanosomiasis, nine strains of Trypanosoma brucei rhodesiense were acquired and used to establish mouse models of African trypanosomiasis which will be suitable for testing drug protocols against both early and late stage disease. Experiments were performed to determine the maximum tolerated difluoromethylornithine (DFMO) dose. DFMO was used to treat groups of mice infected with different strains of T. brucei rhodesiense at the early stage of infection. Variation was found in the sensitivity of the various strains to DFMO as predicted. A range of combinations of DFMO and suramin were administered to uninfected mice in order to detect any possible toxic interactions. No were found.

20. DISTRIBUTION / AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS	Unclassified

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL
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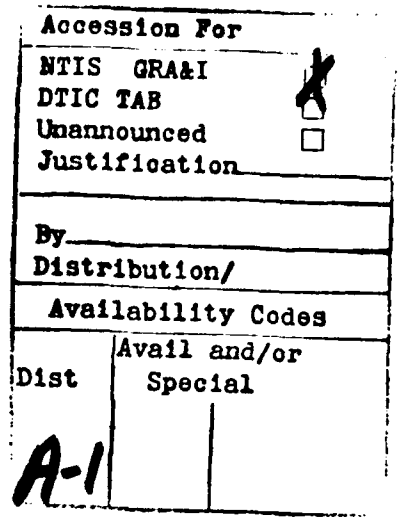
DD Form 1473, JUN 86 *Previous editions are obsolete.* SECURITY CLASSIFICATION OF THIS PAGE



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SUMMARY

OVERVIEW OF PROJECT: The general objective of this contract is to provide a preclinical evaluation of the potential of DFMO in combination with suramin as a treatment of human Trypanosoma brucei rhodesiense infections. Since this disease progresses rapidly, a useful therapy must be active against both the early and late stages of the disease; i.e., both prior to and after the central nervous system (CNS) becomes infected. Therefore the tools used to evaluate efficacy of the therapy are mouse models of both early and late stage T. b. rhodesiense sleeping sickness. Several strains of parasites are used in the models in order to have an initial gauge of the range of sensitivities that might be expected in the field. The project also includes a study designed to detect potential adverse interactions between these two drugs.

SUMMARY OF PROGRESS IN THE FIRST YEAR: Towards these goals, we have: acquired 9 strains of Trypanosoma brucei rhodesiense and established mouse models which will be suitable for testing drug protocols against both early and late stage disease (obj. 1); performed the experiments to determine the maximum tolerated DFMO dose (obj. 2); used DFMO to treat groups of mice infected with different strains of T. brucei rhodesiense at the early stage of infection and found that the strains do vary in sensitivity as predicted (obj 4 and 7); administered a range of combinations of DFMO and suramin to uninfected mice in order to detect any possible toxic interactions and found none (obj 9). Objectives 3 and 10 of the proposal were not accepted as part of the contract and were not pursued. A portion of objectives 4 and 7 as well as objectives 5 and 6 remain to be done in the second year. Progress is on the schedule presented in the proposal.

FOREWORD

Citations of commercial organizations or trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal resources Commission of Life Sciences, National Research Council (NIH Publication No. 86-23, Revised 1985).

BODY OF REPORT

INTRODUCTION:

Present capabilities to treat cases of African sleeping sickness are inadequate. This is especially true of those cases caused by Trypanosoma brucei rhodesiense. Therefore more efficacious and less toxic drugs are needed. The ultimate objective is to develop a new and effective treatment based on interference with polyamine function of these parasites.

Studies with the exquisitely specific polyamine biosynthesis inhibitor DL-alpha-difluoromethylornithine (DFMO, eflornithine, Ornidyl®) showed this drug to cure mice infected with T. brucei brucei (Bacchi, et al., 1980; McCann et al., 1984). Initial (Sjoerdsma and Schechter, 1984) and subsequent extensive clinical trials (Schechter et al., 1987) have shown it to cure patients infected with T. brucei gambiense (West African or Gambian sleeping sickness) in both early (before the central nervous system, CNS, has become involved) and late cases (after the parasites have invaded the CNS). However, in the T. brucei brucei-mouse model of CNS disease, DFMO does not produce a 100% cure rate (Clarkson, et al., 1984). The limited number T. brucei rhodesiense (East African or Rhodesian sleeping sickness) cases treated with DFMO has indicated that DFMO is not as effective for T. brucei rhodesiense as it is for T. brucei gambiense. According to Dr. Peter deRaadt, who is responsible for the trypanosomiasis section of the World Health Organization TDR program, about a dozen T. brucei rhodesiense patients were treated with DFMO in Zimbabwe and most were not cured. In Kenyan trials against T. brucei rhodesiense using twice the dose of DFMO as used for T. brucei gambiense infections, only 3 of 7 T. brucei rhodesiense patients treated with DFMO have been cured. There is only one report on DFMO treatment of a mouse model of T. brucei rhodesiense but with that strain of T. brucei rhodesiense in the mouse, DFMO was successful (McCann et al., 1981).

Previous work has shown that DFMO acts synergistically with other anti-trypanosome drugs (Bacchi et al., 1982; Clarkson et al., 1983; Clarkson et al., 1984). For example, the inability of DFMO to cure late stage T. brucei brucei infections in mice can be overcome by using DFMO in combination with either the anticancer drug bleomycin (Clarkson et al., 1983) or with suramin, an established trypanocidal drug (Clarkson et al., 1984). These combinations provide a 100% cure rate in mice with late stage T. brucei brucei disease although neither bleomycin nor suramin alone, like DFMO, will cure these animals. The combination of drugs also allows the dose of each drug to be greatly reduced compared to the dose when used singly.

The enhanced action of a combination of DFMO with another trypanocidal drug offers a real possibility of extending DFMO therapy to patients infected with T. brucei rhodesiense. A combination of DFMO, which is well tolerated by patients at the doses given for T. brucei gambiense, with suramin is more promising than one with bleomycin. Bleomycin is a toxic and expensive drug that would not be easily used under the conditions sleeping sickness treatment is given. Suramin, however, is generally well tolerated despite sporadic incidences of toxicity. The dose of suramin required for successful combination with DFMO is only 4% of the total suramin dose (mg/kg) used for human non-CNS disease. Therefore the combination of suramin and DFMO may well provide a safe and effective treatment for all stages of T. brucei rhodesiense infection.

The work reported here is progress made in a feasibility study undertaken to investigate the potential of the combination of suramin and DFMO for treatment of early and late stage T. brucei rhodesiense. This is a preclinical study utilizing mouse models of early and late stage disease. Since the response of T. brucei rhodesiense-infected patients has not been consistent, this study uses multiple stocks of T. brucei rhodesiense isolated from patients in East Africa to determine the spectrum of sensitivity to the drugs singly and in combination. The study includes preliminary experiments designed to detect any obvious adverse interaction between the two drugs.

METHODS:

PARASITE STRAINS: Six frozen T. brucei rhodesiense stocks which had been isolated from sleeping sickness patients in East Africa were obtained from the Kenyan Institute for Trypanosomiasis Research. These are designated KETRI 2772, KETRI 2285, KETRI 2537, KETRI 2562, KETRI 2482 and KETRI 2545. In addition a strain from the American Type Culture Collection, ATCC 30119, was used.

STABILATE PREPARATION: Frozen parasites were thawed and inoculated into rats. The parasitemia reached a level between 10^7 and 10^8 after 3 days to 2 weeks depending on the strain. At that time blood was collected and diluted 8 fold with a 1:1 mixture of fetal bovine serum (FBS) and phosphate buffered saline with glucose (PSG - 0.10 mM sodium PO_4, 0.073 mM NaCl, 80 mM glucose, pH 8.0) and held at 0° C. A 20% solution of glycerol was prepared in 1:1 FBS/PSG. A volume of the glycerol solution equal to the volume of the diluted blood was added slowly to the diluted blood; the blood was held on ice and gently stirred as the glycerol solution was added. The glycerol/blood mixture was dispensed into cryo vials and held in a -30° freezer for 24 hours then transferred to liquid nitrogen. The stabilate was tested for infectivity after 30 days in liquid nitrogen.

PARASITEMIA EVALUATION AND SAMPLE COLLECTION: For parasitemia detection, a wet mount was prepared from tail blood and examined with phase contrast optics. The parasitemia was estimated using the method of Herbert and Lumsden (1976). An animal was considered negative if no parasites were found in 50 fields.

If tissues from an animal were to be histiologically evaluated for toxic effects after drug administration, the tissues were collected after sacrifice by over-anesthetizing with diethyl ether. Tissue samples with a maximum thickness of 4 mm were immersed in buffered 10% formalin. The formalin was changed after an hour. The samples were sealed in vials. Standard histological processes were used for dehydration, embedding, sectioning and staining with eosin and hematoxylin.

For serum DFMO determinations, blood was collected and allowed to clot. The serum was separated and mixed with 50% trichloroacetic acid (TCA); one part TCA to 4 parts serum. After holding the sample for several hours at 0° to allow the proteins to precipitate, the samples were centrifuged in a microfuge and the supernatant collected for subsequent DFMO analysis by HPLC. This analysis was done as a collaboration with Dr. Cyrus Bacchi of Pace University in his laboratory at no cost to this contract.

DFMO ADMINISTRATION: A solution of the required DFMO concentration was prepared in tap water and presented to the animals as their drinking water. The water was changed 3 times a week and at no time were the animals without DFMO-containing water.

SURAMIN ADMINISTRATION: Suramin was prepared as a 4 mg/ml solution in 0.85% NaCl and injected into a tail vein at the indicated dose schedule.

RESULTS:

MAXIMUM TOLERATED DFMO DOSE IN UNINFECTED ANIMALS: Ten mice were randomly assigned to each of the following groups: a control group given no treatment and experimental groups administered DFMO in the drinking water at 2%, 3%, 4% and 5%. Treatment was continued for 6 weeks. The mice were then sacrificed and blood and tissues collected for analysis.

No adverse effects were observed during the treatment period in any group. There were no deaths and no diarrhea or other signs of debilitation. Statistical analyses on weekly body weight data collected are in process but there was no obvious difference in weight gain in any group. Preliminary histological results returned from Dr. Schwartz, the pathology consultant, indicate that only a slight blunting of the intestinal villi could be seen at the highest dose. Otherwise he has observed no difference in tissues from any of the groups. HPLC analysis for DFMO in the serum samples was done on a collaborative basis in Dr. Cyrus Bacchi's laboratory. The chromatograms are being analyzed to determine the actual DFMO content of the serum after six weeks administration. Published data on human serum DFMO concentration vs dose will be compared to the mouse serum concentration vs dose. This will allow a direct determination of the therapeutic effect of DFMO in the mouse models of T. brucei rhodesiense sleeping sickness at a clinically relevant serum DFMO concentration.

LACK OF ADVERSE EFFECTS OF THE COMBINATION OF DFMO AND SURAMIN: Two series of mice were administered suramin in a graded dose schedule. In each series of nine groups, sequential suramin daily injections were given for 0, 1, 2, 3, 4, 5, 6, 7 or 8 days. Each suramin dose was 40 mg/kg i.v. to yield total doses of 0, 40, 80, 120, 160, 200, 240, 280 and 320 mg/kg, respectively. Each group contained 10 randomly assigned mice. One series was also administered 2% DFMO in the drinking water beginning with the initiation of the suramin treatment and continuing until the time of sacrifice. The other series was given no DFMO. The animals were sacrificed 14 days after the last suramin dose. In the series given DFMO, the group of mice given no suramin was treated with 2% DFMO for 14 days and then sacrificed.

In the entire experiment there was one spontaneous death. This occurred in the group given 2% DFMO and a total surami. dose of 200 mg/kg. Since this is in the middle of the suramin dose range and the death occurred 9 days after the last suramin dose, this death cannot be attributed to suramin/DFMO toxicity.

There were no signs of diarrhea in any of the treated animals nor any fur ruffling which is virtually always seen in sick or debilitated mice. Statistical analysis of the hematocrits taken at the time of sacrifice is being done but inspection of the data indicates no reduction in packed cell volume in any group relative to the controls. Similarly, statistical analysis of mouse weight gain during the treatment period is in process but inspection reveals no difference from one series to the other or in groups within a series. A pooled 24 hour urine sample was collected just from each group before sacrifice; no group showed any proteinuria which would be expected if DFMO enhanced suramin nephrotoxicity, the most common toxic effect of suramin.

SENSITIVITY OF MULTIPLE STOCKS OF T. brucei rhodesiense TO DFMO: Seven stocks of T. b. rhodesiense were established and tested for DFMO sensitivity. Groups of 5 mice were inoculated with 25,000 parasites and the groups were

treated 24 hours after inoculation. Treatment was with 2% or 4% DFMO in the drinking water for 3, 6 or 9 days. The data for the acute models (Figure 1) show that this series of parasite stocks exhibits a range of sensitivities to DFMO. Figure 2 presents the same data except with grouping by treatment schedule and not by parasite stock. Figure 2 shows that treatment with 2% for 6 days provided a much higher cure rate than treatment with 4% for 3 days although the total amount of DFMO administered was similar. This emphasizes the importance of the duration of treatment over total dose. Although the sensitivity of the various T. brucei rhodesiense strains to DFMO differed, if adequate dosage were given all strains could be treated successfully with DFMO in the acute stage.

ESTABLISHMENT OF A LATE STAGE MODEL OF T. brucei rhodesiense: A total of seven strains of T. brucei rhodesiense were tested for the ability to create a chronic infection of sufficient duration to allow late stage or CNS disease to develop. Figures 3 - 10 present the course of parasitemias and survival times for mice infected with various size inocula of the T. brucei rhodesiense stocks. Of the seven, three (KETRI 2537, KETRI 2545 and KETRI 2285) allowed the mice to live for the 3 weeks that insures CNS involvement (Jennings et al., 1977; Murray and Jennings, 1983). Therefore three T. brucei rhodesiense CNS models have been developed and these can be used for testing suramin/DFMO. The other strains were much more acute; three killed within a week even with an inoculum of only 10 parasites. For one strain there was a difference in the course of parasitemias depending on the inoculum size in that the lowest inoculum did not establish an infection (KETRI 2545, Figure 6). The inoculum size had no effect for the other strains.

DISCUSSION:

The purpose of the experiment designed to determine the maximum tolerated dose of DFMO in mice was to be able to reproduce a dose regimen equivalent to that used in man. The underlying assumption was that since DFMO is being used for T. brucei rhodesiense-infected patients at the maximum tolerated dose, then the maximum tolerated dose in mice should produce an equivalent pharmacological activity. The experiment was done as contracted but since no intolerance was observed, one cannot assume that the maximum tolerable dose was reached. However, because serum samples were collected, DFMO analysis was possible. This was done on a collaborative basis with Dr. Cyrus Bacchi (Haskins Laboratories, Pace University, New York, NY) in his laboratory and without additional cost being added to the contract. When the data has been analyzed and the mouse DFMO dosage/serum concentration profile compared to published human DFMO dosage/serum concentration data, a valid comparison of the DFMO serum concentration and cure rate of mice and men will be possible. This data will also be useful in interpreting the mouse CNS model response to suramin+DFMO with respect to probable clinical utility.

There were no apparent toxic effects when suramin was combined with DFMO despite the very high dosage of suramin. While there is very little data on suramin toxicity in the literature, the little information available indicated that the maximum dose of 320 mg/kg would be well above the LD_{50} in mice (Merck Index mouse suramin LD_{50} = 40 mg/kg i.v. which is equal to the individual daily doses given the mice). The pathology report is not complete but the observed equal weight gain in all groups, lack of proteinuria, maintenance of condition, equivalence of hematocrits and absence of diarrhea all indicate a lack of toxicity. This is especially encouraging when the maximum mouse dose of 320 mg suramin over 9 days is compared to the full human therapeutic dose of 75 mg/kg over 3 weeks. Although small animals often both require and tolerate higher drug doses than larger animals, this is usually due to the faster drug clearance rate of small animals. However, since suramin binds to albumin and is cleared only slowly in both small and large animals, dose responses to suramin, both therapeutic and adverse, are not likely to be extremely different.

The various T. brucei rhodesiense stocks show a range of sensitivity to DFMO. This reflects the clinical situation in that some T. brucei rhodesiense patients have been cured by DFMO and others have not. Eventually, many additional stocks will have to be tested to define more accurately the range of DFMO sensitivity that can be expected in the field. It should be noted that all the tested stocks will respond to DFMO if it is used at adequate dosage for an adequate period.

Three T. brucei rhodesiense stocks that can be expected to produce CNS disease in mice have been identified. These will allow the testing of the combination of DFMO with suramin against a model of T. brucei rhodesiense late stage disease.

In summary, the basic work on toxicity and model development has been completed. This will allow the testing of the combination of the efficacy of the combination of suramin and DFMO against T. brucei rhodesiense during the second year.

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FIGURE 1

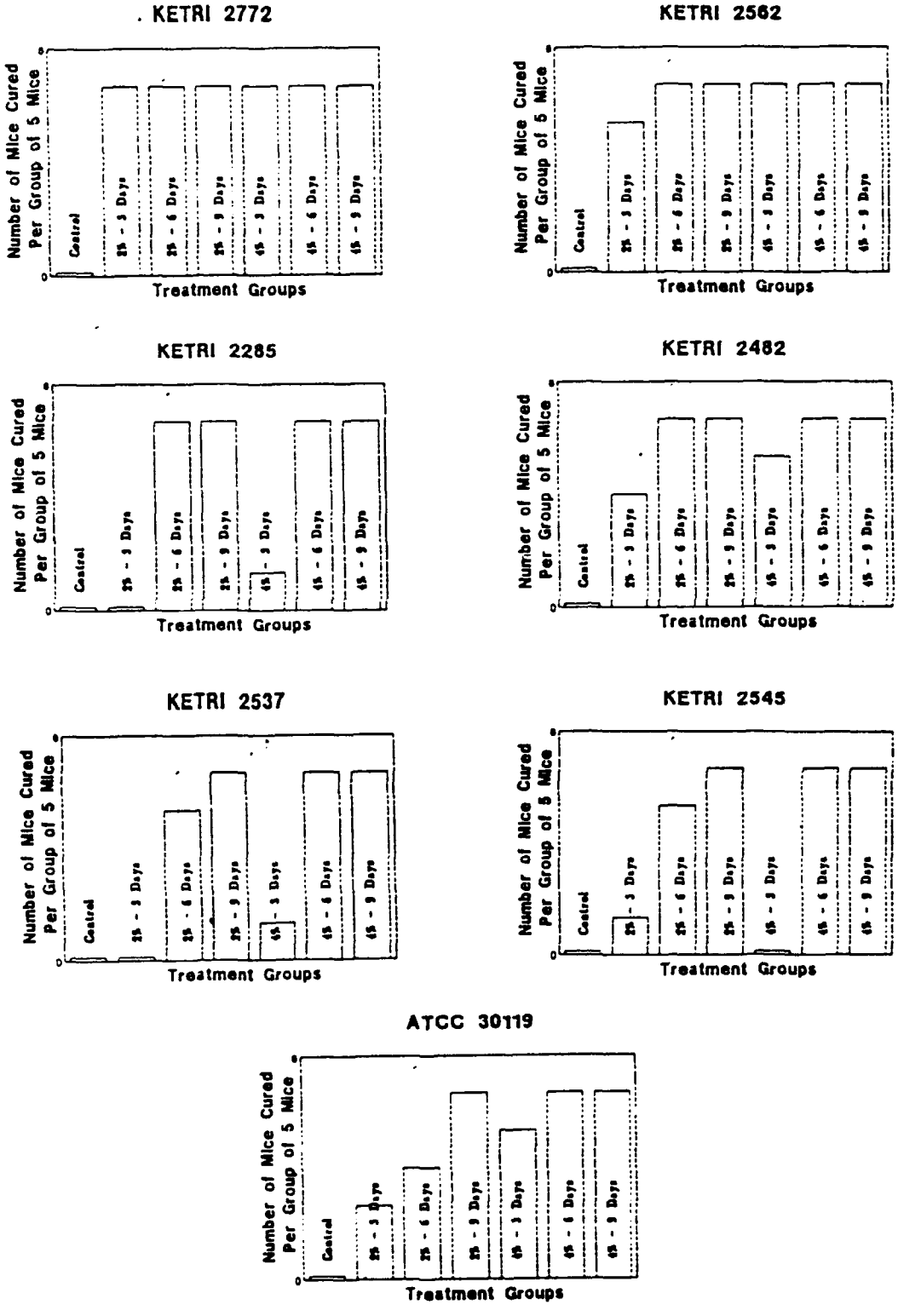
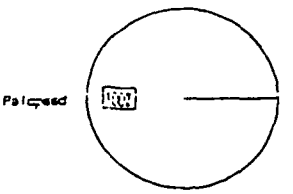

KETRI 2772
KETRI 2562
KETRI 2285
KETRI 2482
KETRI 2537
KETRI 2545
ATCC 30119
Number of Mice Cured Per Group of 5 Mice
Treatment Groups
Control
25 - 3 Days
25 - 6 Days
25 - 9 Days
45 - 3 Days
45 - 6 Days
45 - 9 Days

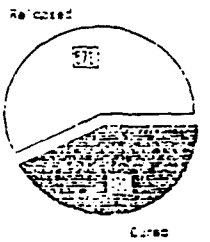
FIGURE 2

TRYPANOSOMA BRUCEI RHODESIENSE
Control Groups



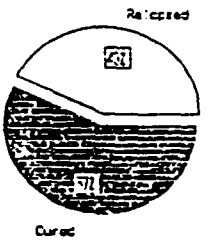
April-May 1988

TRYPANOSOMA BRUCEI RHODESIENSE
2% DFMO in Drinking Water for 3 Days



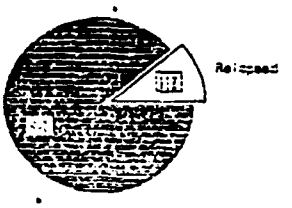
April-May 1988

TRYPANOSOMA BRUCEI RHODESIENSE
4% DFMO in Drinking Water for 3 Days



April-May 1988

TRYPANOSOMA BRUCEI RHODESIENSE
2% DFMO in Drinking Water for 6 Days



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TRYPANOSOMA BRUCEI RHODESIENSE
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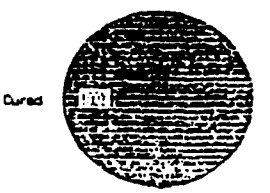

April-May 1988

TRYPANOSOMA BRUCEI RHODESIENSE
2% DFMO in Drinking Water for 9 Days

April-May 1988

TRYPANOSOMA BRUCEI RHODESIENSE
4% DFMO in Drinking Water for 9 Days

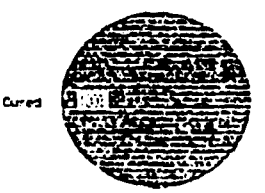

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FIGURE 3

KETRI 2537

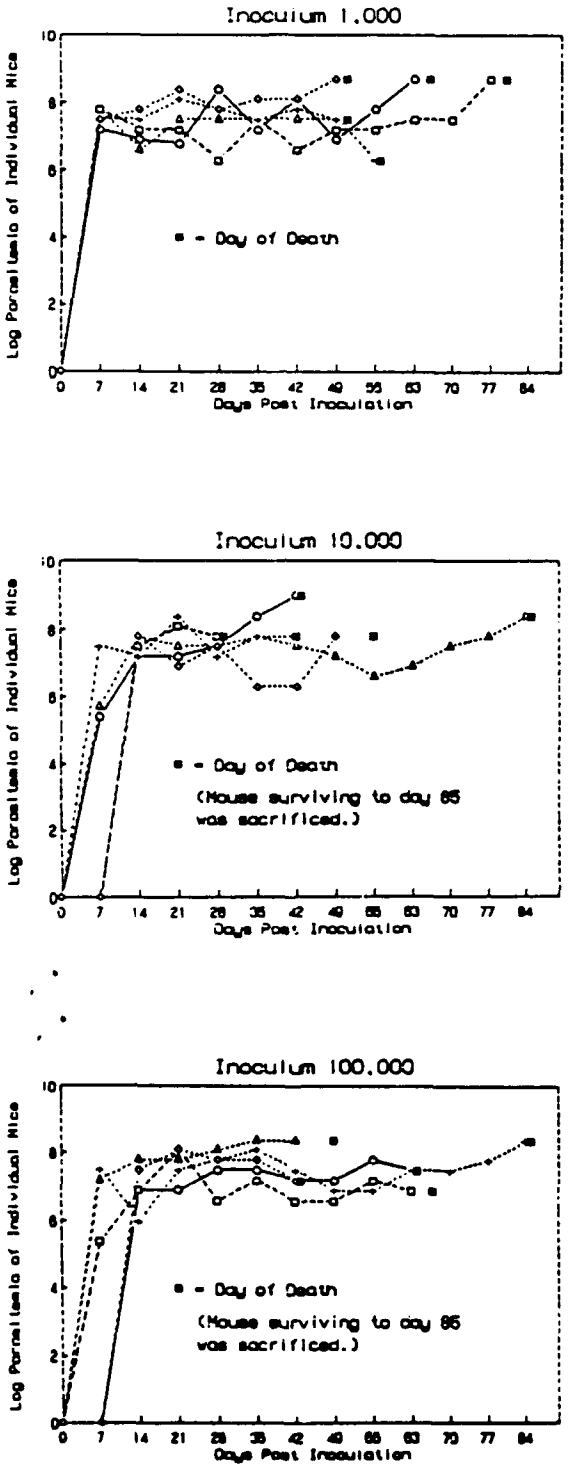
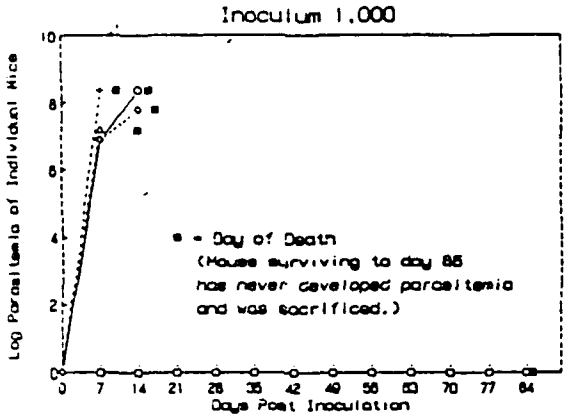
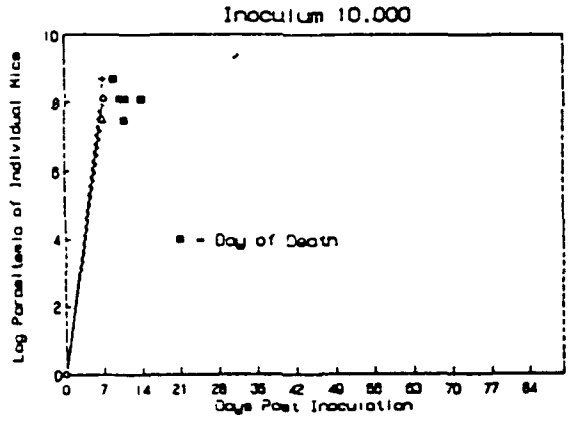


FIGURE 4

KETRI 2482





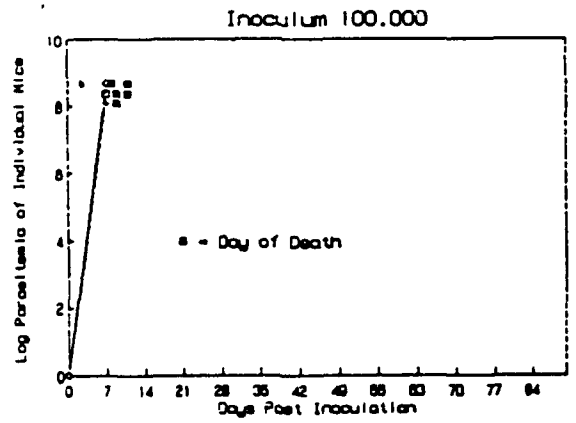


FIGURE 5

KETRI 2285

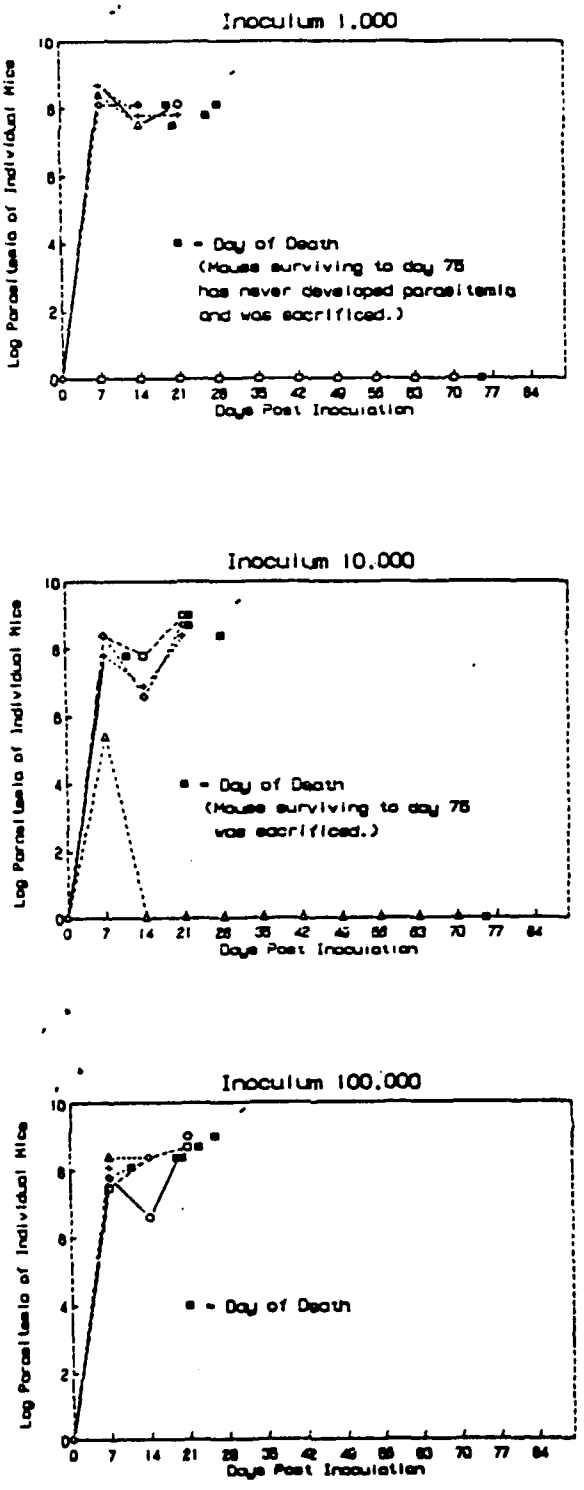
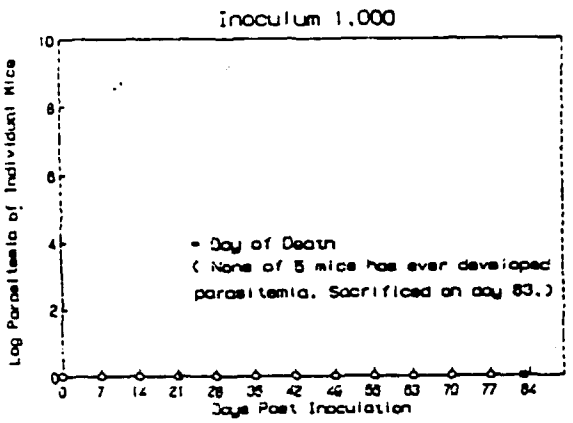
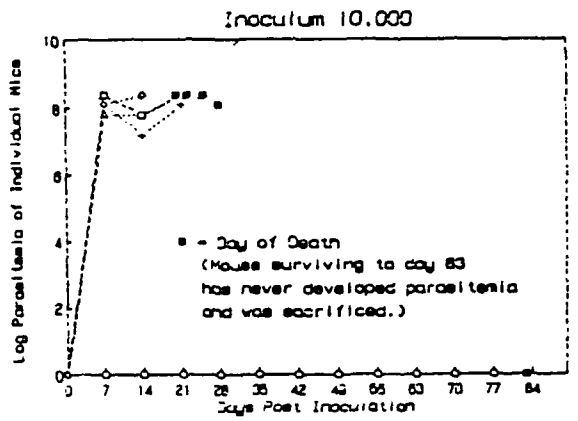


FIGURE 6

KETRI 2545





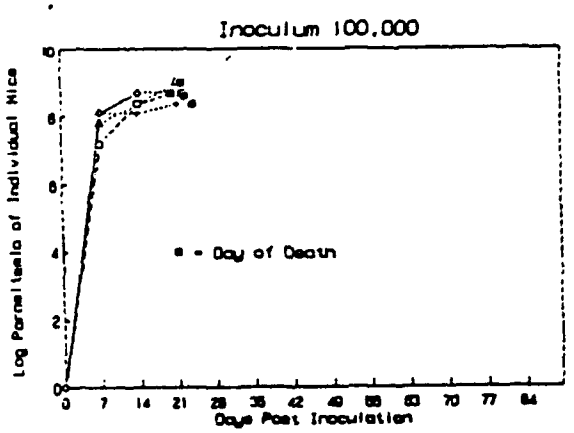
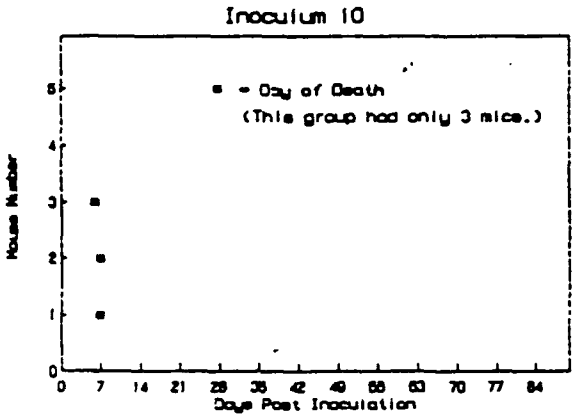
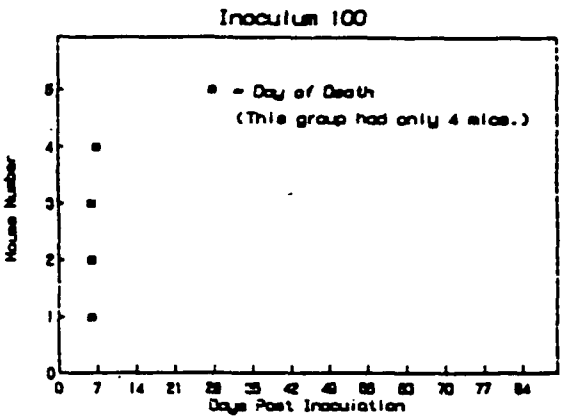
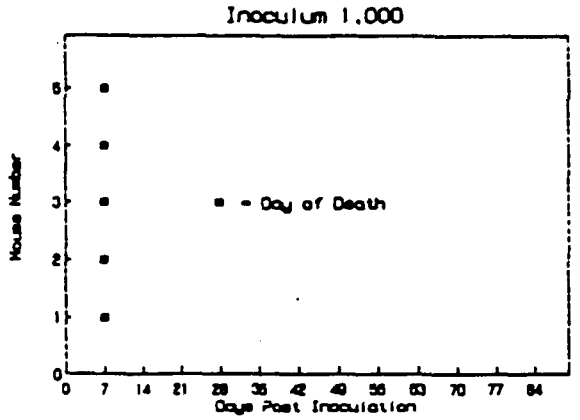


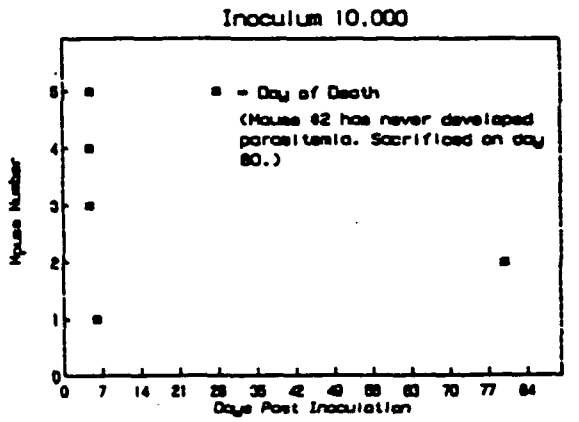
FIGURE 7

KETRI 2772









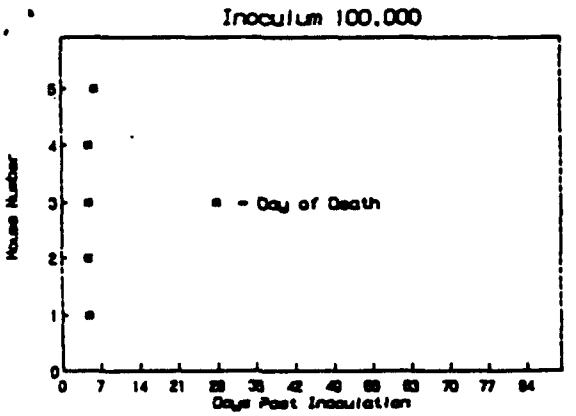


FIGURE 8

ATCC 30119

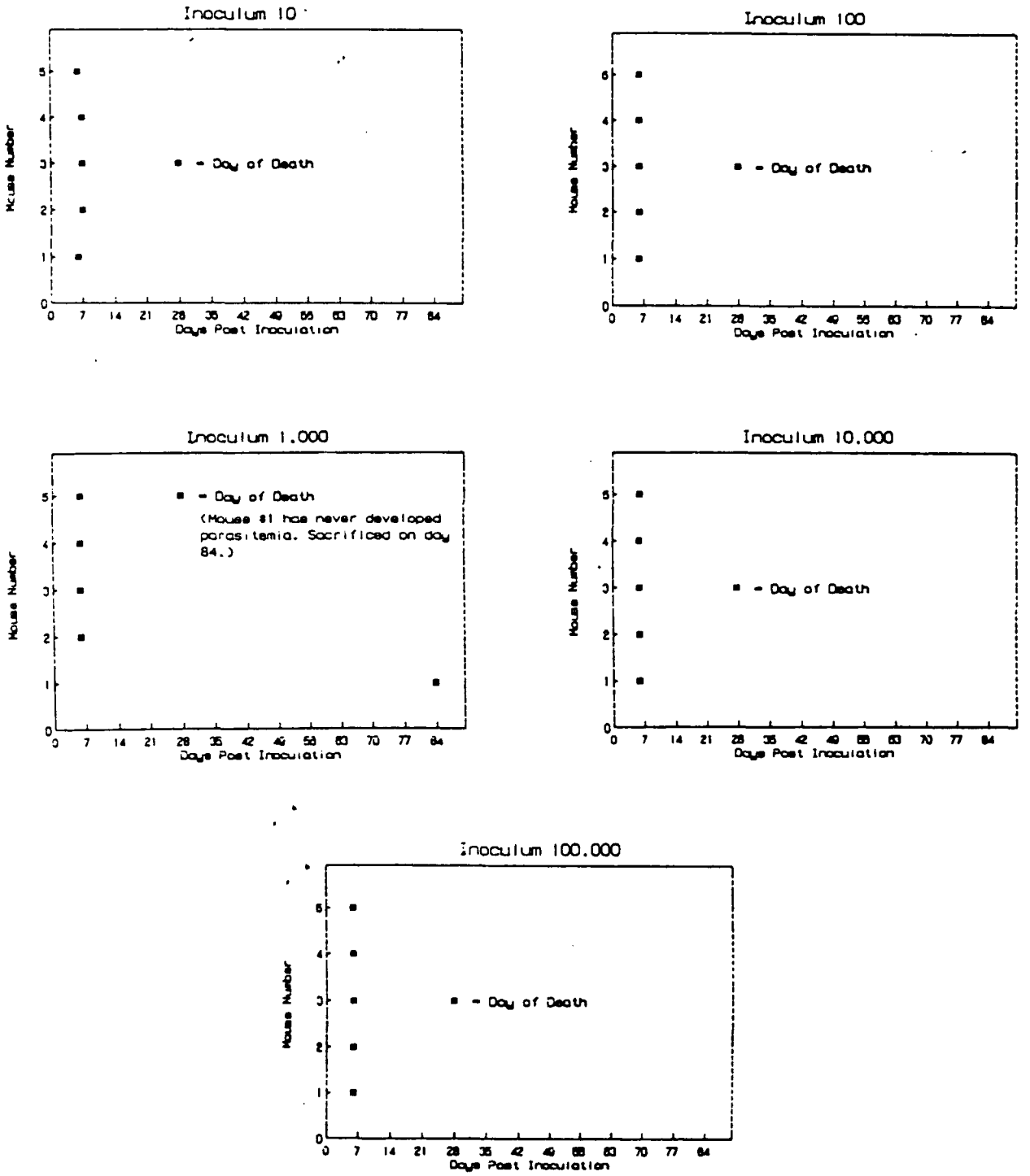


FIGURE 9

KETRI 2562